Dkt. #639-C-PCT-US

Application for United States Letters Patent

To all whom it may concern:

Be it known that **Nai-Kong CHEUNG**

Has invented certain new and useful improvements in

THERAPY-ENHANCING GLUCAN

of which the following is a full, clear and exact description.

THERAPY-ENHANCING GLUCAN

This application is a continuation-in-part of U.S. Serial No. 10/621,027, Filed July 16, 2003, the contents of which are incorporated in its entirety by reference here into this application.

Throughout this application, various references are cited. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains.

BACKGROUND OF THE INVENTION

This disclosure relates to a method for introducing substances into cells comprising contacting a composition comprising orally administered beta-glucan with said cells. A feature of this invention provides a method for introducing substances into a subject comprising administering to the subject an effective amount of the above compositions. The substance which could be delivered orally includes but is not limited to peptides, proteins, RNAs, DNAs, chemotherapeutic agents, biologically active agents, and plasmids. Other small molecules and compounds may be used as well. Another feature of the present invention is a composition comprising orally administered beta-glucan capable of enhancing efficacy of IgM antibodies.

Glucans derived from cell walls of yeasts, such as Saccharomyces cervisiae or mutant yeast strains described in U.S. Patent No. 5,250,436, the disclosure of which is incorporated herein in its entirety by reference, may be used in the above compositions. Glucans having β(1-3) and β(1-6) linkages may be prepared by the process described in U.S. Patent No. 5,233,491 and 4,810,646, the disclosures of which are incorporated herein in their entirety by reference. Soluble or aqueous glucans which are suitable for oral administration may be produced by the process described in U.S. Patent No. 4,810,646 and 5,519,009, the

disclosures of which are incorporated herein in their entirety by reference.

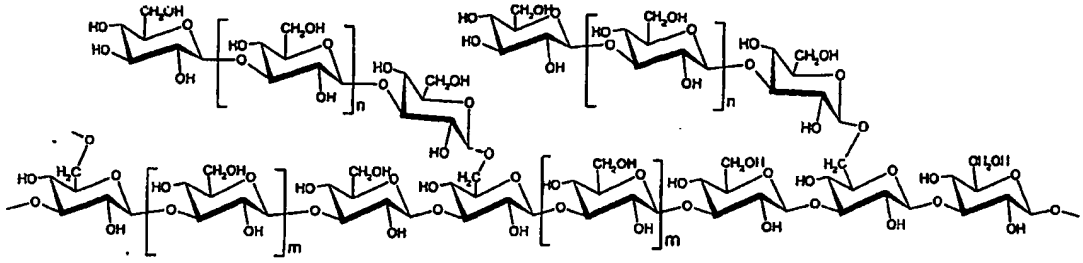
Beta-glucans have been tested for tumor therapy in mice for nearly 40 years.[1,2] Several forms of mushroom derived beta-glucans are used clinically to treat cancer in Japan, including PSK (from Coriolus versicolor), Lentinan and Schizophyllan. In randomized trials in Japan, PSK has moderately, but significantly improved survival rates in some cancer trials: after gastrectomy,[3,4] colorectal surgery,[5,6] and esophagectomy[7] to remove primary tumors. Results have been less encouraging in breast cancer,[8,9] and leukemia.[10] Schizophyllan has improved survival of patients with operable gastric cancer,[11] inoperable gastric cancer,[12,13] and cervical cancer.[14] Again, though survival differences between groups were statistically significant, these improvements were modest. While beta-glucans are not widely used by Western oncologists, beta-glucan containing botanical medicines such as Reishi and maitake[15] are widely used by U.S. cancer patients as alternative/complementary cancer therapies. These previous studies that looked for a therapeutic effect of beta-glucan, did not incorporate co-administration of therapeutic monoclonal antibodies (MoAb) as part of the protocol. There is increasing evidence that antibody is necessary to deposit iC3b which acts as a potent opsonin of human tumors. When beta-glucan is administered without co-administration of MoAb, its tumor cytotoxic effect requires the presence of naturally-occurring antitumor antibodies which can be quite variable among patients and even in experimental mice.

Anti-tumor effect of beta-glucan when combined with cancer specific antibodies was previously described. Previous studies have shown that oral beta-glucans derived from barley or oats can greatly enhance the anti-tumor activity of anti-tumor

monoclonal antibodies in xenograph models. See Therapy-Enhancing Glucan, Int'l Application No. PCT/US02/01276, filed January 15, 2002. Cheung et al., Oral (1-3),(1-4)-beta-glucan syngergizes with anti-ganglioside GD2 monoclonal antibody 3F8 in the therapy of neuroblastoma. Clin Cancer Res. 2002;8:1217-1223. Cheung NK et al., Orally administered beta-glucans enhance anti-tumor effects of monoclonal antibodies. Cancer Immunol Immunother. 2002; 51:557-564. The phase I clinical trial supports the prediction that barley beta-glucan can enhance the antibody effect on metastatic cancer. As previously noted, lentinan and laminarin, both (1→3),(1→6)-β-D-glucans, were not as effective as barley glucan.[16] In addition, among the (1→3),(1→4)-β-D-glucans, small molecular weight preparations and Lichenans were not as effective. The molecular size and the fine structure of beta-glucan may have substantial influence on their synergistic effect on antibodies towards tumors.

In Europe and USA beta-glucans especially from Bakers' yeast have long been employed as feed additives for animals, as dietary supplement for humans,[17] in treatment of wounds,[18] and as an active ingredient in skin cream formulations. The basic structural unit in beta-glucans is the β(1→3)-linked glucosyl units. Depending upon the source and method of isolation, beta-glucans have various degrees of branching and of linkages in the side chains. The frequency and hinge-structure of side chains determines its immunomodulor effect. beta-glucans of fungal and yeast origin are normally insoluble in water, but can be made soluble either by acid hydrolysis or by derivatisation introducing charged groups like -phosphate, -sulphate, -amine, -carboxymethyl and so forth to the molecule.[19,20]

Soluble glucan with the molecular structure where (1→3)-β-D-glucan units form the backbone with branches made up of (1→3)-β-D-glucan units positioned at (1→6)-β-D-glucan hinges was isolated from Baker's yeast, *Saccharomyces cerevisiae*. High molecular weight fractions were obtained and



tested for synergy with monoclonal antibodies in tumor models. The anti-tumor effect of soluble yeast beta-glucan was found to be comparable to the anti-tumor effect of soluble barley beta-glucan, when combined with monoclonal antibodies specific for human cancer as detailed below.

SUMMARY OF THE INVENTION

This invention provides a method for introducing substances into cells comprising contacting a composition comprising orally administered beta-glucan with said cells.

Another aspect of the present is a method for introducing substances into a subject comprising administering to the subject an effective amount of the above compositions. The substance which could be delivered orally includes but is not limited to peptides, proteins, RNAs, DNAs, chemotherapeutic agents, biologically active agents, and plasmids. Other small molecules and compounds may be used as well.

A further aspect of the present invention is a composition comprising orally administered beta-glucan capable of enhancing efficacy of IgM antibodies.

DETAILED DESCRIPTION OF THE FIGURES

Figure 1. Barley (1→3),(1→4)-β-D-glucan plus antibody in the treatment of metastatic neuroblastoma in patients. MIBG scan before and after treatment in a patient with metastatic neuroblastoma refractory to multiple regimens of chemotherapy. Patient received intravenous anti-GD2 antibody 3F8 (10 mg/m2/day) for a total of 10 days, plus oral barley beta-glucan over the same time period. Figure 1A shows baseline MIBG scan of patient. Extensive osseous metastasis can be seen in the femora, fibulae, pelvis, ribs, left scapula, right clavicle, humeri, skull and spine. Heart, liver, stomach and colon uptakes are physiologic. Figure 1B shows MIBG scan of same patient 2 months later, following a single cycle of therapy with 3F8 plus glucan. Areas of metastases have significantly improved.

Figure 2. Barley (1→3),(1→4)-β-D-glucan plus antibody in treatment of subcutaneous human lymphoma xenografted in SCID mice. SCID mice with established subcutaneous Daudi (n=9) (Fig. 2A), Hs445 (n=5) (Fig. 2B), EBV-derived LCL (n=9) (Fig. 2C) and RPMI 6666 (n=10; data not shown) xenografts were treated either with 200ug intravenous rituximab twice weekly for 8 doses (■),400ug (1→3), (1→4)-D-β-glucan administered orally via intragastric gavage daily for 29 days (Δ) or a combination of rituximab and (1→3), (1→4)-D-β-glucan (x), or left untreated (◆). Percentage tumor growth is plotted on y-axis and days after treatment was commenced on x-axis. Error bars represent SEM and have been shown only for rituximab alone and combination groups. For all xenografts, only combination treatment was associated with reduction in tumor growth. The reduction in tumor growth per day in the group receiving beta-glucan in addition to rituximab compared to rituximab alone was 2.0% (95% CI 1.3-2.7%; $p<0.0005$) for Daudi, 0.8% for EBV-derived LCL (95% CI 0.4-1.2%; $p<=001$), 2.2% for Hs445 (95% C.I. 1.2%-3.2%; $p=0.0009$), and 1.8% for RPMI6666 (95% CI 1.0-2.7 %; $p<0.0002$; data not shown)

xenografts.

Figure 3. Barley (1→3),(1→4)-β-D-glucan plus antibody in treatment of disseminated human lymphoma xenografted in SCID mice. $5x10^6$ Daudi (Fig. 3A) or Hs445 (Fig. 3B) cells in 100 μl normal saline were injected intravenously (IV) into SCID mice. Mice were treated either with 200ug intravenous rituximab twice weekly for 8 doses (---), 400ug (1→3), (1→4)-D-β-glucan administered orally via intragastric gavage daily for 29 days (...) or a combination of rituximab and (1→3), (1→4)-D-β-glucan (——), or left untreated (——) commencing 10 days after tumor implantation. Tumors grew systemically and mice became paralyzed when tumor cells infiltrated the spinal canal, resulting in hind-leg paralysis. Mice were sacrificed at onset of paralysis or when animals lost 10% of their body weight. Kaplan-Maier survival curves for the various groups are shown in Figures 2A (Daudi) and 2B (Hs445). Mice treated with a combination of (1→3), (1→4)-D-β-glucan and rituximab had a significantly increased survival when compared to all other treatment groups ($p<0.0005$ for Daudi and $p=0.001$ for Hs445) or when compared to rituximab alone ($p<0.0005$ for Daudi and $p=0.01$ for Hs445). Median survival for mice with no treatment, rituximab alone, BG, and rituximab+BG groups was 27,71,43 and 124 days respectively for Daudi xenografts, and 12, 16, 31 and 243 days respectively for Hs445 xenografts.

Figure 4. Dose response of 3G6 (anti-GD2 IgM antibody) in the presence of barley β-glucan in the treatment of human neuroblastoma. Two million LAN1 neuroblastoma cells were xenografted subcutaneously in athymic Balb/c mice. Treatment started in groups of 5 mice each, 2 weeks after tumor implantation when visible tumors reached 0.7-0.8 cm diameter. 3G6 group (solid squares) was treated with 200 ug of intravenous 3G6 injected through the retroorbital plexus twice weekly (M and Th). 3G6 + BG group was treated with 200 ug i.v. 3G6 twice weekly plus

oral beta-glucan (BG) 400 ug daily by gavage for a total of 14-18 days. 3G6 was administered in 3 different doses (open triangle 8 ug per dose, open square 40 ug, open circle 200 ug). BG group (solid circles) received 400 ug oral beta-glucan alone. Tumor size was measured from the first day of treatment, and the product of the largest diameters expressed as percent of the size on day 0 of treatment. Vertical bars represent standard errors, depicted in only 4 groups for clarity. While BG alone and 3G6 alone showed no anti-tumor effect, the BG+200 ug 3G6 group showed highly significant tumor shrinkage and suppression which was 3G6 dose-dependent ($p<0.05$).

Figure 5. Treatment of human neuroblastoma using 3G6 (anti-GD2 IgM antibody) in the presence of yeast (1→3),(1→6)-β-D-glucan. Two million LAN1 neuroblastoma cells were xenografted subcutaneously in athymic Balb/c mice. Treatment started in groups of 5 mice each, 2 weeks after tumor implantation when visible tumors reached 0.7-0.8 cm diameter. 3G6 group (solid squares) was treated with 200 ug of intravenous 3G6 injected through the retroorbital plexus twice weekly (M and Th) for a total of 5 doses. Particulate yeast glucan group (solid triangles) received 400 ug oral particulate yeast glucan alone. 3G6 + whole yeast particles (open diamond) was treated with 200 ug iv 3G6 twice weekly plus yeast particles 400 ug daily by gavage for a total of 14-18 days. 3G6 + soluble yeast glucan group was treated with 200 ug iv 3G6 twice weekly plus soluble yeast glucan 400 ug daily by gavage for a total of 14-18 days. 3G6 + particulate yeast glucan group was treated with 200 ug i.v. 3G6 twice weekly plus particulate yeast glucan 400 ug daily by gavage for a total of 14-18 days. Tumor size was measured from the first day of treatment, and the product of the largest diameters expressed as percent of the size on day 0 of treatment. Vertical bars represent standard errors, depicted in only 4 groups for clarity. While glucan alone and 3G6 alone showed no anti-tumor effect,

soluble and particulate yeast glucan when combined with 3G6 group showed highly significant tumor shrinkage and suppression ($p<0.05$).

Figure 6. Treatment of human neuroblastoma using 3F8 (anti-GD2 IgG antibody) in the presence of barley and yeast β-glucan. Two million LAN1 neuroblastoma cells were xenografted subcutaneously in athymic Balb/c mice. Treatment started in groups of 5 mice each, 2 weeks after tumor implantation when visible tumors reached 0.7-0.8 cm diameter. 3F8 group (solid diamonds) was treated with 200 ug of intravenous 3F8 injected through the retroorbital plexus twice weekly (M and Th) for a total of 5 doses. Barley glucan group (solid squares) received 400 ug barely glucan alone. 3F8 + barley glucan group (open diamond) was treated with 200 ug i.v. 3F8 twice weekly plus barely glucan 400 ug daily by gavage for a total of 14-18 days. 3F8 + soluble yeast glucan group (open squares) was treated with 200 ug iv 3F8 twice weekly plus soluble yeast glucan 400 ug daily by gavage for a total of 14-18 days. Tumor size was measured from the first day of treatment, and the product of the largest diameters expressed as percent of the size on day 0 of treatment. Vertical bars represent standard errors. While glucan alone and 3F8 alone showed no anti-tumor effect, barley and soluble yeast glucan when combined with 3F8 group showed highly significant tumor shrinkage and suppression ($p<0.05$).

Figure 7. Treatment of disseminating human lymphoma in SCID mice using Rituxan and barley or yeast β-glucan. 5x10e6 Daudi cells in 100 μl normal saline were injected intravenously (IV) into SCID mice. Tumors grew systemically and mice became paralyzed when tumor cells infiltrated the spinal canal, resulting in hind-leg paralysis. Mice were sacrificed at onset of paralysis or when animals lost 10% of their body weight. Therapy was initiated ten days after injection of tumor cells. 40µg rituximab (Genentech, San Francisco, CA) was injected

intravenously twice weekly for a total of eight injections and 400μg glucan administered orally via intragastric gavage daily for 29 days. Mice were weighed weekly and observed clinically at least once daily. Mice receiving rituxan plus barley glucan or rituxan plus yeast soluble glucan have a highly significant prolonged survival ($p<0.05$).

Figure 8 illustrates the pEGP-C1 vector purchased from BD Biosciences (Palo Alto, CA).

Figure 9 shows glucan facilitates gene transfer into monocytes.

Figure 10 illustrates higher molecular weight β-glucan and gene transfer.

Figure 11 illustrates presence of GFP mRNA in circulating monocytes.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides a composition for oral uptake of substance comprising an appropriate amount of carbohydrates. In an embodiment, the carbohydrate is glucan.

When administered orally, glucan is taken up by macrophages and monocytes which carry these carbohydrates to the marrow and reticuloendothelial system from where they are released, in an appropriately processed form, onto myeloid cells including neutrophils, and onto lymphoid cells including natural killer (NK) cells. This processed glucan binds to CR3 on these neutrophils and NK cells, activating them in tumor cytotoxicity in the presence of tumor-specific antibodies.

Since macrophage and monocytes ingest glucan (whether soluble, gel or particle) from the gut, glucan is a potential conduit for

gene therapy. Unlike proteins, DNA or plasmids are relatively heat-stable, and can be easily incorporated into warm soluble barley glucan which gels when cooled to room or body temperature. When mice are fed these DNA-glucan complexes, reporter genes can be detected in peripheral blood monocytes and macrophages within days. More importantly these reporter genes are expressed in these cells, a few days after ingestion of these DNA complexes. These findings have potential biologic implications. Glucan and similar carbohydrates may be conduits for DNA or plasmids to get into the human body. Oral glucan may be a convenient vehicle for correcting genetic defects of macrophages/monocytes, or administering genetic vaccines.

As it can easily be appreciated by an ordinary skilled artisan, other carbohydrates capable of functioning like glucan could be identified and used in a similar fashion. One easy screening for such carbohydrates can be established using glucan as the positive control.

The glucan includes but is not limited to β(1-3) and β(1-4) mixed linkage-glucan, and the glucan is of high molecular weight. The glucan may also have β(1-3) and β(1-6) linkages.

This invention also provides a method for introducing substance into cells comprising contacting the above compositions with said cells. One can use reporter genes or other markers to assess the efficiency of the said introduction. Reporter genes or markers are well known in the molecular biology field. In addition, this invention provides a method for introducing substance into a subject comprising administering to the subject an effective amount of the above compositions.

This invention provides a composition for the oral delivery of one or more substances comprising an effective amount of an

orally administered beta-glucan and one or more chemotherapeutic agents.

In an embodiment, the glucan contains 1,3-1,6 or 1,3-1,4 mixed linkages, or a mixture of 1,3-1,6 and 1,3-1,4 mixed linkages. In another embodiment, the glucan enhances the efficacy of chemotherapeutic agents or anti-cancer antibodies.

In a further embodiment, the glucan is derived from grass, plants, mushroom, yeast, barley, fungi, wheat or seaweed. The glucan may be of high molecular weight. The molecular weight of the glucan may be at least 10,000 Daltons.

In a further embodiment, the substance is a peptide, protein, RNA, DNA, plasmid, or chemotherapeutic agent. As used herein, chemotherapeutic agents include chemicals that combat disease in the body of an animal or medications used to treat various forms of cancer.

This invention provides a method for introducing substance into cells comprising contacting the above-described composition with said cells.

The substance which could be delivered orally includes but is not limited to peptides, proteins, RNAs, DNAs, and plasmids. Other small molecules and compounds may be used as well.

This invention provides a method for treating a subject comprising administering to the subject an effective amount of the above composition. In an embodiment, the method further comprises the substance.

This invention provides a method for treating a subject with genetic disorder comprising administering to the subject an effective amount of the above-described composition and a

substance capable of correcting said genetic disorder. The substance includes but is not limited to a peptide, protein, RNA, DNA, plasmid and other small molecule and compound.

This invention provides a composition comprising an effective amount of orally administered (1→3), (1→6) beta-glucan capable of enhancing efficacy of IgM antibodies.

This invention provides a composition comprising an effective amount of orally administered (1→3), (1→6) beta-glucan capable of enhancing efficacy of antibodies. Glucans derived from cell walls of yeasts, such as Saccharomyces cervisiae or mutant yeast strains described in U.S. Patent No. 5,250,436, the disclosure of which is incorporated herein in its entirety by reference, may be used in the above compositions. Glucans having β(1-3) and β(1-6) linkages may be prepared by the process described in U.S. Patent No. 5,233,491 and 4,810,646, the disclosures of which are incorporated herein in their entirety by reference. Soluble or aqueous glucans which are suitable for oral administration may be produced by the process described in U.S. Patent No. 4,810,646 and 5,519,009, the disclosures of which are incorporated herein in their entirety by reference.

In an embodiment, the antibody is a monoclonal antibody, or an antibody against cancer or tumor cells, which include but are not limited to anti-CEA antibody, anti-CD20 antibodies, anti-CD25 antibodies, anti-CD22 antibodies, anti-HER2 antibodies, anti-tenascin antibodies, MoAb M195, Dacluzimab, anti-TAG-72 antibodies, R24, Herceptin, Rituximab, 528, IgG, IgM, IgA, C225, Epratuzumab, and MoAb 3F8. In another embodiment, the antibody is a tumor-binding antibody.

Moreover, the antibody is capable of activating complement and/or activating the antibody dependent cell-mediated

cytotoxicity. In another embodiment, the antibody modulates T-cell or B-cell function.

In a further embodiment, the antibody is directed at the epidermal growth factor receptor, a ganglioside, such as GD3 or GD2.

In a further embodiment, the antibodies are effective against cancers which include neuroblastoma, melanoma, non-Hodgkin's lymphoma, Epstein-Barr related lymphoma, Hodgkin's lymphoma, retinoblastoma, small cell lung cancer, brain tumors, leukemia, epidermoid carcinoma, prostate cancer, renal cell carcinoma, transitional cell carcinoma, breast cancer, ovarian cancer, lung cancer, colon cancer, liver cancer, stomach cancer, or other gastrointestinal cancers.

In a further embodiment, the above-described composition is in a pharmaceutically acceptable carrier.

This invention provides a method for treating a subject comprising administrating the above-described composition to a subject.

This invention provides a composition comprising an effective amount of orally administered (1→3), (1→6) beta-glucan capable of enhancing efficacy of vaccines. In an embodiment, the vaccine is against cancer or infectious agents, such as bacteria, viruses, fungi, or parasites.

This invention provides a composition comprising an effective amount of orally administered (1→3), (1→6) beta-glucan capable of enhancing efficacy of natural antibodies or infectious agents.

This invention provides a composition comprising an effective amount of orally administered (1→3),(1→6) beta-glucan capable of enhancing host immunity.

This invention provides a composition comprising an effective amount of orally administered (1→3),(1→6) beta-glucan capable of enhancing the action of an agent in preventing tissue rejection. In an embodiment, the tissue is transplanted tissue or transplanted organ or the host as in graft-versus-host disease.

In an embodiment, the glucan of the above-described composition has high molecular weight. The molecular weight of glucan is at least 10,000 Daltons. In another embodiment, the glucan is derived from barley, oat, mushroom, seaweed, fungi, yeast, wheat or moss. In a further embodiment, the glucan is stable to heat treatment.

In a further embodiment, above-describe composition is stable after boiling for 3 hours. The effective dose of the above-described composition is about >= 25 mg/kg/day, five days a week for a total of 2-4 weeks.

The invention will be better understood by reference to the Experimental Details which follow, but those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative, and are not meant to limit the invention as described herein, which is defined by the claims which follow thereafter.

Example I

Phase I study of barley β-glucan in combination with anti-GD2 antibody in stage 4 neuroblastoma.

A total of 24 patients were studied. These patients are all children or adolescents with relapsed or refractory stage 4neuroblastoma metastatic to bone, marrow or distant lymph nodes, some with large soft tissue masses. Beta-glucan was well tolerated with no dose-limiting toxicities. Anti-tumor responses were recorded for marrow disease (histology, MIBG scans), soft tissue tumors (CT), as well biochemical markers (urine VMA and HVA tumor markers). One example of tumor response is shown in figures 1A and 1B: ^{131}I-metaiodobenzylguanidine (MIBG) scans showing near-complete resolution of extensive metastases after one treatment cycle of 3F8 plus beta-glucan. These responses are uncommon in patients with refractory or relapsed metastatic stage 4 NB treated with 3F8 alone or 3F8 in combination with cytokines. The best response rate for 3F8 to date was in a Phase II trial of combination 3F8 plus GMCSF where 7 of 33 (21%) children achieved MIBG improvement. In contrast, 62% (13 of 21) evaluable patients on 3F8 + beta-glucan had MIBG improvement, a near tripling of the response rate ($p=0.008$ by χ^2). In addition, among 15 patients with marrow disease, 5 achieved complete marrow remission (30%), and 8 with stable disease in the marrow.
(See Fig. 1)

Example II

Rituximab activates complement-mediated and antibody-dependent cell-mediated cytotoxicities, and is effective against B-cell lymphomas. Beta-glucans are naturally occurring glucose polymers that bind to the lectin domain of CR3, a receptor widely expressed among leukocytes, priming it for binding to iC3b

activated by antibodies. Barley-derived (1→3),(1→4)-β-D-glucan (BG), when administered orally (400 µg per day x 29 days), strongly synergized with subtherapeutic doses of intravenous rituximab (200 µg twice/week x 8 doses) in the therapy of CD20-positive human lymphomas. Growth of established subcutaneous non-Hodgkin's lymphoma (NHL) (Daudi and EBV-derived B-NHL) or Hodgkin's disease (Hs445 or RPMI6666) xenografted in SCID mice was significantly suppressed, when compared to mice treated with rituximab or BG alone. Survival of mice with disseminated lymphoma (Daudi and Hs445) was significantly increased. There was no weight loss or clinical toxicity in treated animals. This therapeutic efficacy and lack of toxicity of BG plus rituximab supports further investigation into its clinical utility.

Introduction

The chimeric anti-CD20 antibody rituximab is being evaluated in an increasing number of disorders. After clinical efficacy was initially demonstrated against relapsed and refractory follicular/low grade non-Hodgkin's lymphoma[1], responses to rituximab have been reported in other malignant and non-malignant B-cell disorders[2]. Several mechanisms of action have been proposed including activation of apoptotic pathways[3], elaboration of cytokines[4], and elicitation of host complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC)[5]. Although many patients with B-cell disorders respond to rituximab, remissions are often transient[6]. More than 50% of lymphomas recurrent after rituximab treatment failed to respond the second time[7]. Mechanisms of resistance to rituximab are as yet unclear, and may include paucity or loss of target antigen[8], pharmacokinetic variations among individual patients, FcR polymorphism[9], resistance to complement activity[10], or inherent gene expression of the lymphoma[11].

beta-glucans are complex polymers of glucose with affinity for the lectin site of the CR3 receptor on leucocytes[12]. With bound beta-glucan, CR3 (CD11b) is primed to engage iC3b fragments deposited on cells by complement-activating antibodies. This receptor mediates the diapedesis of leukocytes through the endothelium and stimulates phagocytosis, degranulation and tumor cytotoxicity. Many fungi present beta-glucan or beta-glucan-like CR3 binding ligands on their cell surface. Hence, when iC3b deposition occurs, both CD11b and lectin sites become engaged, and phagocytosis and respiratory burst is triggered[13]. In contrast, tumor cells lack such molecules, and even when coated with iC3b do not generally activate CR3 and cannot activate leucocytes. Soluble forms of beta-glucan bind to lectin sites and prime both phagocytic and NK cells to kill iC3b-coated tumor targets[14].

(1→3), (1→4)-D-β-glucan (BG), a soluble, barley-derived beta-glucan has advantages over previously studied (1→3), (1→6)-β-glucans, particularly efficacy when administered orally and a good safety profile[15]. In vivo synergism between BG and the complement-fixing antibody 3F8 against human neuroblastoma xenografts[15,16] was recently demonstrated. The synergism between BG and rituximab against lymphoma is now reported.

Study Design

Cell lines:

Human Burkitt's lymphoma cell line, Daudi, and Hodgkin's disease (HD) cell lines Hs445 and RPMI 6666 were purchased from American Type Culture Collection (Rockville, MD). Human EBV-BLCL were established using previously described methods[17].

Mice:

Fox Chase ICR SCID mice (Taconic, White Plains, NY) were maintained under institutionally approved guidelines and protocols.

Tumor models:

Subcutaneous tumors were established by injecting $5x10^6$ cells suspended in 0.1 ml of Matrigel (Becton-Dickinson, Franklin Lakes, NJ) into mice flanks. Tumor dimensions were measured two to three times a week and tumor size was calculated as the product of the two largest diameters. Mice were sacrificed when maximum tumor dimension exceeded 20mm. A disseminated tumor model was established in SCID mice as previously described[18]. Briefly, $5x10^6$ Daudi or Hs445 cells in 100 μl normal saline were injected intravenously into SCID mice. Tumors grew systemically and mice became paralyzed when tumor cells infiltrated the spinal cord, resulting in hind-leg paralysis. Mice were sacrificed at onset of paralysis or when animals lost 10% of their body weight.

Treatment regimens:

For mice with subcutaneous tumors, therapy was initiated after tumors were established (7-8mm diameter). For the disseminated tumor model, therapy was initiated ten days after injection of tumor cells. Groups of at least five mice per treatment regimen received either rituximab, BG, neither or both. 200µg rituximab (Genentech, San Francisco, CA) was injected intravenously twice weekly for a total of eight injections and 400µg BG (Sigma, St. Louis, MO) administered orally via intragastric gavage daily for 29 days. Animals were weighed weekly and observed clinically at least once daily.

Statistical analysis:

Tumor growth was calculated by fitting a regression slope for each individual mouse to log transformed values of tumor size. Slopes were compared between groups using t-tests using a previously described method for censored observations[19]. Survival in mice with disseminated disease was compared using Kaplan-Meier analysis and proportion of deaths was compared by Fisher's exact $\chi 2$ test. Analyses were conducted using STATA 7

Results and Discussion

In all subcutaneous xenograft models, significant reduction in tumor growth was noted in mice treated with a combination of rituximab and BG. Mice treated with rituximab alone showed a modest reduction in tumor growth, while those treated with BG alone or left untreated had unabated tumor growth (Figure 1A, 1B, 1C). All tumors except for those treated with combination therapy grew beyond 20 mm size and mice had to be sacrificed. Mice on combination treatment had persistent tumor suppression even after treatment was stopped. In a multivariable linear model of tumor growth rate, using dummy variables for treatment, the interaction between BG and rituximab was positive and significant, demonstrating synergism.

For disseminated xenografts, there was a significant difference in survival between the combination and control groups for both NHL and HD models ($p<0.005$, by log-rank) (Figure 2). 5/38 mice and 2/8 mice with disseminated Daudi and Hs445 tumors respectively treated with combination BG and rituximab were surviving >12 months after therapy was discontinued suggesting complete eradication of disease. In contrast, 0/29 and 0/8 mice receiving rituximab alone in respective groups survived (15% vs. 0% survival; $\chi 2=0.01$). There was no significant weight loss or other clinically apparent adverse effects. That BG is absorbed can be inferred from the fact that it could be detected intracellularly within fixed and permeabilized peripheral blood leucocytes by immunofluorescence (data not shown).

In these studies, synergism between BG and rituximab was highly significant irrespective of the type of CD20-positive lymphoma. Improved responses in Daudi xenografts as compared to Hs445 may be attributable to higher CD20 expression in the former (Mean geometric fluorescence channel for Daudi 241 compared to 184 for

Hs445). When tumors that progressed were examined for CD20 expression by immunofluorescence studies of single cell suspensions or indirect immunohistochemistry of frozen sections, no significant difference was noted between groups treated with rituximab, BG alone or rituximab+BG (data not shown), indicating that treatment with rituximab+BG was not associated with loss of CD20.

Synergism between other complement-activating monoclonal antibodies and BG[15,16] were previously demonstrated. The current data extend this observation to rituximab. CDC is considered an important mechanism for rituximab cytotoxicity. Rodent complement is not inhibited efficiently by human complement regulatory proteins (mCRP). Therefore CDC can be an effective anti-tumor mechanism in xenograft models. However in a study, at sub-therapeutic doses of antibody, rituximab-mediated ADCC and CDC were not sufficient to effect tumor cell killing. Since BG has no direct effect on ADCC[20], this synergy is most likely a result of iC3b-mediated tumor cytotoxicity. Lymphoma cells express mCRP including CD46, CD55, and CD59[10,21]. However, iC3b-mediated cytotoxicity is unaffected by the presence of CD59 which affects only MAC-mediated complement cytotoxicity[22]. Furthermore, in human breast carcinoma tumors, deposition of iC3b has been demonstrated despite the presence of mCRP[23] indicating that unlike their inhibitory effect on MAC, effect on iC3b-mediated tumor cytotoxicity is not absolute.

If this synergistic effect can be safely reproduced in humans, iC3b-mediated cytotoxicity may be a potential strategy to overcome rituximab resistance in patients with B-cell malignancies. Since neither T nor B cells are required for this synergistic effect, BG may have a potential role even in immunocompromised lymphoma patients. Furthermore, in patients with autoimmune disorders, B-cell depletion may be enhanced with this non-toxic oral therapy. Conversely, beta-glucans can

enhance release of cytokines such as TNF-α and IL-6[24], and because the acute toxicities of rituximab are also related to cytokine release secondary to complement activation[25], there is a potential of increased toxicity when BG and rituximab are used in combination. Carefully designed phase I studies are necessary in order to define the safety and efficacy in developing BG as an adjunct to rituximab therapy in the treatment of B-cell disorders and in antibody-based therapies of other cancers.

References for Example II

1. Maloney DG, Liles TM, Czerwinski DK, Waldichuk C, Rosenberg J, Grillo-Lopez A, Levy R. Phase I clinical trial using escalating single-dose infusion of chimeric anti-CD20 monoclonal antibody (IDEC-C2B8) in patients with recurrent B-cell lymphoma. Blood. 1994;84:2457-2466

2. Cheson BD. Rituximab: clinical development and future directions. Expert Opin Biol Ther. 2002;2:97-110

3. Alas S, Emmanouilides C, Bonavida B. Inhibition of interleukin 10 by Rituximab results in Down-regulation of Bcl-2 and sensitization of B-cell Non-Hodgkin's lymphoma to apoptosis. Clin Cancer Res. 2001;7:709-723

4. Chow KU, Sommerlad WD, Boehrer S, Schneider B, Seipelt G, Rummel MJ, Hoelzer D, Mitrou PS, Weidmann E. Anti-CD20 antibody (IDEC-C2B8, rituximab) enhances efficacy of cytotoxic drugs on neoplastic lymphocytes in vitro: role of cytokines, complement, and caspases. Haematologica. 2002;87:33-43

5. Reff ME, Carner K, Chambers KS, Chinn PC, Leonard JE, Raab R, Newman RA, Hanna N, Anderson DR. Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20. Blood. 1994;83:435-445

6. McLaughlin P, Grillo-Lopez AJ, Kink BK, Levy R, Czuczman MS, Williams ME, Heyman MR, Bence-Bruckler I, White CA, Cabanillas F, Jain V, Ho AD, Lister J, Wey K, Shen D, Dallaire BK. Rituximab chimeric anti-CD20 monoclonal antibody therapy for relapsed indolent lymphoma: half of patients respond to four-dose treatment program. J Clin Oncol. 1998;16:2825-2833

7. Davis TA, Grillo-Lopez AJ, White CA, McLaughlin P, Czuczman MS, Link BK, Maloney DG, Weaver RL, Rosenberg J, Levy R. Rituximab anti-CD20 monoclonal antibody therapy in non-Hodgkin's lymphoma: safety and efficacy of re-treatment. J Clin Oncol. 2000;18:3135-3143

8. Davis TA, Czerwinski DK, Levy R. Therapy of B-cell lymphoma with anti-CD20 antibodies can result in the loss of CD20 antigen expression. Clin Cancer Res. 1999;5:611-615

9. Cartron G, Dacheux L, Salles G, Solal-Celigny P, Bardos P, Colombat P, Watier H. Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcgammaRIIIa gene. Blood. 2002;99:754-758

10. Golay J, Zaffaroni L, Vaccari T, Lazzari M, Borleri GM, Bernasconi S, Tedesco F, Rambaldi A, Introna M. Biologic response of B lymphoma cells to anti-CD20 monoclonal antibody rituximab in vitro: CD55 and CD59 regulate complement-mediated cell lysis. Blood. 2000;95:3900-3908

11. Bohen SP, Troyanskaya OG, Alter O, Warnke R, Botstein D, Brown PO, Levy R. Variation in gene expression patterns in follicular lymphoma and the response to rituximab. Proc Natl Acad Sci U S A. 2003;100:1926-1930

12. Bohn JA, BeMiller JN. (1-3)-B-D-Glucans as biological response modifiers:a review of structure-functional activity

relationships. Carbohydr Polymers. 1995;28:3-14

13. Ross GD, Cain JA, Myones BL, Newman SL, Lachmann PJ. Specificity of membrane complement receptor type three (CR3) for beta-glucans. Complement Inflamm. 1987;4:61-74

14. Xia Y, Vetvicka V, Yan J, Hanikyrova M, Mayadas T, Ross GD. The beta-glucan-binding lectin site of mouse CR3 (CD11b/CD18) and its function in generating a primed state of the receptor that mediates cytotoxic activation in response to iC3b-opsonized target cells. J Immunol. 1999;162:2281-2290

15. Cheung NK, Modak S. Oral (1-3),(1-4)-beta-glucan syngergizes with anti-ganglioside GD2 monoclonal antibody 3F8 in the therapy of neuroblastoma. Clin Cancer Res. 2002;8:1217-1223

16. Cheung NK, Modak S, Vickers A, Knuckles B. Orally administered beta-glucans enhance anti-tumor effects of monoclonal antibodies. Cancer Immunol Immunother. 2002;51:557-564

17. Koehne G, Gallardo HF, Sadelain M, O'Reilly RJ. Rapid selection of antigen-specific T lymphocytes by retroviral transduction. Blood. 2000;96:109-117

18. Wei BR, Ghetie MA, Vitetta ES. The combined use of an immunotoxin and a radioimmunoconjugate to treat disseminated human B-cell lymphoma in immunodeficient mice. Clin Cancer Res. 2000;6:631-642

19. Vardi Y, Ying Z, Zhang C-H. Two-sample tests for growth curves under dependent right censoring. Biometrika. 2001;88:949-960

20. Yan J, Vetvicka V, Xia Y, Coxon A, Carroll MC, Mayadas TN, Ross GD. B-glucan a "Specific" biologic response modifier that uses antibodies to target tumors for cytotoxic recognition by leukocyte complement receptor type 3 (CD11b/CD18). J Immunol. 1999;163:3045-3052

21. Treon SP, Mitsiades C, Mitsiades N, Young G, Doss D, Schlossman R, Anderson KC. Tumor cell expression of CD59 is associated with resistance to CD20 serotherapy in patients with B-cell malignancies. J Immunother. 2001;24:263-271

22. Jurianz K, Ziegler S, Garcia-Schuler H, Kraus S, Bohana-Kashtan O, Fishelson Z, Kirschfink M. Complement resistance of tumor cells: basal and induced mechanisms. Mol Immunol. 1999;36:929-939

23. Vetvicka V, Thornton BP, Wieman TJ, Ross GD. Targeting of natural killer cells to mammary carcinoma via naturally occurring tumor cell-bound iC3b and beta-glucan-primed CR3 (CD11b/CD18). J Immunol. 1997;159:599-605

24. Adachi Y, Okazaki M, Ohno N, Yadomae T. Enhancement of cytokine production by macrophages stimulated with (1-->3)-beta-D-glucan, grifolan (GRN), isolated from Grifola frondosa. Biol Pharm Bull. 1994;17:1554-1560

25. Van der Kolk LE, Grillo-Lopez AJ, Baars JW, Hack CE, van Oers MH. Complement activation plays a key role in the side-effects of rituximab treatment. Br J Haematol. 2001;115:807-811

Example III

Barley β-glucan extract synergizes with IgM antibodies

Natural IgM antibody from human serum when administered i.v. was cytotoxic for human neuroblastoma (NB) cells effecting growth arrest of subcutaneous solid human NB xenografts in nude rats. (1, 2) IgM was taken up by the tumors with massive perivascular complement activation and accumulation of granulocytes after 24 hours. (3) In metastatic NB model, IgM antibody was effective in eliminating tumors in 90% of the mice. (4) The absence of this anti-NB IgM antibody during infancy and among NB patients (of any age), and its prevalence after 12 months of age has raised the hypothesis that natural IgM antibodies could play a role as an immunological control mechanism against NB. (5) 3G6 is an anti-GD2 mouse IgM monoclonal antibody (MoAb). Within 48 hours after i.v. injection of biotinylated 3G6, subcutaneous NB xenografts showed membrane staining of tumor cells. Although 3G6 had lower mean fluorescence (53 ± 19 fluorescent channel units, n=7 mice) when compared to 3F8, an IgG MoAb (149±44, n=7), 3G6 plus beta-glucan was effective against sc human NB ($p<0.05$), with a dose response curve (Fig. 4) comparable to that of 3F8. (6) These findings were consistent with those using human natural anti-NB IgM. (1, 2) These data support the idea that beta-glucan can enhance not just IgG inducing vaccines, but also IgM inducing vaccines.

References for Example III

1. David K, Ollert MW, Juhl H, et al: Growth arrest of solid human neuroblastoma xenografts in nude rats by natural IgM from healthy humans. Nat Med 2:686-9, 1996

2. Ollert MW, David K, Schmitt C, et al: Normal human serum contains a natural IgM antibody cytotoxic for human neuroblastoma cells. Proc Natl Acad Sci U S A 93:4498-503, 1996

3. Ollert MW, David K, Vollmert C, et al: Mechanisms of in vivo anti-neuroblastoma activity of human natural IgM. Eur J Cancer 33:1942-8, 1997

4. Engler S, Thiel C, Forster K, et al: A novel metastatic animal model reflecting the clinical appearance of human neuroblastoma: growth arrest of orthotopic tumors by natural, cytotoxic human immunoglobulin M antibodies. Cancer Res 61:2968-73, 2001

5. Erttmann R, Schmitt C, Ollert MW, et al: Naturally occurring humoral cytotoxicity against neuroblastoma (NB) cells in healthy persons and NB patients. Pediatr Hematol Oncol 13:545-8, 1996

6. Cheung N, Modak S: Oral (1-3),(1-4)-beta-glucan syngergizes with anti-ganglioside GD2 monoclonal antibody 3F8 in the therapy of neuroblastoma. Clin Cancer Res 8:1217-1223, 2002

Example IV

(1→3),(1→6) β-glucan derived from baker's yeast (derived from Saccharomyces cerevisiae) is also effective in enhancing antibody therapy of cancer

LAN-1 tumor cells were planted ($2x10^6$ cells) in 100 μl of Matrigel (Sigma) subcutaneously. Tumor dimensions were measured two to three times a week with vernier calipers, and tumor size was calculated as the product of the two largest perpendicular diameters. All treatment studies started in groups of 4-5 mice when tumor diameters reached 0.7 to 0.8 cm. Mice received antibody (3F8 or 3G6) treatment (200 ug per day) i.v. (by tail vein injection) twice weekly x 5 doses and oral beta-glucan (400 ug per day) by intragastric injection every day for a total 14-18

days. (See Fig. 5 and 6)

Glucans derived from cell walls of yeasts, such as Saccharomyces cervisiae or mutant yeast strains described in U.S. Patent No. 5,250,436, the disclosure of which is incorporated herein in its entirety by reference, may be used in the above compositions. Glucans having β(1-3) and β(1-6) linkages may be prepared by the process described in U.S. Patent No. 5,233,491 and 4,810,646, the disclosures of which are incorporated herein in their entirety by reference. Soluble or aqueous glucans which are suitable for oral administration may be produced by the process described in U.S. Patent No. 4,810,646 and 5,519,009, the disclosures of which are incorporated herein in their entirety by reference. Beta-glucans such as the Soluble beta-1,3/1,6 glucan or SBG manufactured by Biotec Pharmacon (Norway) may also be used.

In similar experiments a subcutaneous lymphoma model was studied. Here $5x10^6$ cells suspended in 0.1 ml of Matrigel (Becton-Dickinson, Franklin Lakes, NJ) were planted into mice flanks. Tumor dimensions were measured two to three times a week and tumor size was calculated as product of the two largest diameters. Mice were sacrificed when maximum tumor dimension exceeded 20mm. 200μg rituximab (Genentech, San Francisco, CA) was injected intravenously twice weekly for a total of eight injections and 400μg glucan administered orally via intragastric gavage daily for 29 days. Mice were weighed weekly and observed clinically at least once daily. The rate of tumor response and the percent of mice achieving complete remissions were comparable between barley glucan and yeast glucan. These series of subcutaneous tumor models showed that soluble yeast (1→3),(1→6) beta-glucan of large molecular weight (>10,000 Daltons) is equally potent as barley (1→3),(1→4) beta-glucan. In addition, the source and physical form of yeast glucan can

make substantial differences.

Metastatic lymphoma model was also studied. A model of disseminated tumors was established in SCID mice as previously described. (1) Briefly, $5x10^6$ Daudi cells in 100 µl normal saline were injected intravenously (i.v.) into SCID mice. Tumors grew systemically and mice became paralyzed when tumor cells infiltrated the spinal canal, resulting in hind-leg paralysis. Mice were sacrificed at onset of paralysis or when animals lost 10% of their body weight. Therapy was initiated ten days after injection of tumor cells. 40µg rituximab (Genentech, San Francisco, CA) was injected intravenously twice weekly for a total of eight injections and 400µg glucan administered orally via intragastric gavage daily for 29 days. Mice were weighed weekly and observed clinically at least once daily. (See Fig. 7)

Again both barley glucan and yeast glucan showed comparable effect when combined with Rituxan. Neither barely glucan nor yeast glucan has any effect on survival when used alone (data not shown).

References for Example IV

1. Wei BR, Ghetie MA, Vitetta ES: The combined use of an immunotoxin and a radioimmunoconjugate to treat disseminated human B-cell lymphoma in immunodeficient mice. Clin Cancer Res 6:631-642, 2000

Example V

Mechanism by which Orally Administered β-Glucans Function with Anti-Tumor Monoclonal Antibodies to Mediate Tumor Regression. (1)

Using syngeneic tumor (GD2+ RMA-S) in wild type (WT) C57Bl/6 mice versus either CR3-deficient (CD11b -/-) or C3-deficient (C3 -/-) C57Bl/6 mice, MoAb alone elicited no tumor regression, whereas combining the i.v. anti-GD2 MoAb with oral barley or yeast beta-glucan elicited significant regression in WT but not in CR3-deficient mice. Moreover, the combined treatment with i.v. MoAb and oral beta-glucans produced 60-100% tumor-free survivors in WT mice, but only 0-20% survival in the CR3-deficient mice. These experiments demonstrated a near absolute requirement for leukocyte CR3 for the anti-tumor effect, especially when oral barley beta-glucan was given with anti-tumor MoAb. A therapy protocol comparing WT to C3-deficient mice similarly showed that oral beta-glucan therapy required serum C3. When barley beta-glucan and yeast beta-glucan were labeled with fluorescein (BG-F and YG-F) and given to mice by intragastric injection, the trafficking of beta-glucan was followed. Within three days of daily oral administration of BG-F or YG-F, macrophages in the spleen and lymph nodes contained fluorescein-labeled beta-glucan. After 4 d, YG-F and BG-F were also observed in macrophages in bone marrow. When the uptake of YG-F and BG-F by WT versus CR3-deficient mice was compared, no differences were apparent in either the percentage of macrophages containing ingested beta-glucan-F or the amount of beta-glucan-F per cell. Thus, the uptake of barley and yeast beta-glucan by gastrointestinal macrophages does not require CR3 and is likely mediated instead by Dectin-1.(2) Macrophages in vitro and in the marrow were able to degrade large molecules of barley or yeast beta-glucan into smaller biologically-active fragments of beta-glucan that are then released.

To determine if the soluble beta-glucan-F released by macrophages had indeed been taken up by bone marrow granulocytes, groups of WT or CR3-deficient mice that had been given YG-F or BG-F for 10 days were injected i.p. with thioglycolate medium

to elicit the marginated pool of bone marrow granulocytes into the peritoneal cavity. Only WT granulocytes were able to pick up the YG-F and BG-F released from macrophages. These data suggest a sequential ingestion of beta-glucan by gastrointestinal macrophages that shuttle the beta-glucan to the bone marrow where soluble degradation fragments are released and taken up by granulocytes via membrane CR3. When peritoneal granulocytes were isolated from WT and CR3-deficient mice that had been given oral beta-glucan, only WT granulocytes were able to kill iC3b-coated tumor cells in vitro. These experiments show that bone marrow granulocytes and tissue macrophages acquire membrane CR3-bound soluble beta-glucan from gastrointestinal macrophages, and that this bound beta-glucan primes the CR3 of both granulocytes and macrophages so that when they are recruited to a site of inflammation they are able to kill iC3b-coated tumor cells.

References for Example V

1. Hong F, Yan J, Baran JT, et al: Mechanism by which orally administered beta(1,3)-glucans function with anti-tumor monoclonal antibodies to mediate tumor regression and tumor-free survival. J Exp Med, 2004

2. Herre J, Gordon S, Brown GD: Dectin-1 and its role in the recognition of beta-glucans by macrophages. Mol Immunol 40:869-76, 2004

Example VI

Soluble β-glucan can be used as a conduit for plasmids.

The major obstacles for the delivery of DNA, RNA and proteins orally are the acidic and proteolytic environment of the stomach, and limited uptake of proteins by the GALT. It is believed that M cells within the Peyer's patches and phagocytes are the predominant vehicles for uptake of microparticulates. However,

nanoparticles may also access GALT via a paracellular mechanism [1,2] and by transcytosis.[3] In either case, particle uptake observed can be improved using particles with mucoadhesive properties or affinity for receptors on cells. Many polymers have been used to fabricate nanoparticles are mucoadhesive. Among them are alginate, carrageenans, and pectin. Although these materials were often used as the core polymers in nanoparticulates, no specific receptor has been identified for these polymers and the efficiency of uptake remains suboptimal. Dectin-1 is now known to be a universal receptor for β-glucan, and is found in many human tissues including monocytes and phagocytes. The gelling properties of high molecular weight β-glucan allows RNA, DNA and proteins to be embedded. Since sugars are highly resistant to acid conditions and enzymes, proteins, RNA and DNA remain protected during their passage through the gastrointestinal tract. Through the high affinity Dectin-1 receptor for β-glucan, these substances can be introduced into the phagocytes as potential vehicles to the rest of the body.

The pEGP-C1 vector (See Fig. 8) was purchased from BD Biosciences (Palo Alto, CA) and prepared according to manufacturers' instructions. pEGFP-C1 encodes a red-shifted variant of wild-type GFP (1-3) which has been optimized for brighter fluorescence and higher expression in mammalian cells. (Excitation maximum = 488 nm; emission maximum = 507 nm.) The vector backbone also contains an SV40 origin for replication in mammalian cells only if they express the SV40 T-antigen. A bacterial promoter upstream of this cassette expresses kanamycin resistance in E. coli. The pEGFP-C1 backbone also provides a pUC origin of replication for propagation in E. coli and an f1 origin for single-stranded DNA production.

Mice were fed with 50 μg pEGFP-c1 plasmid mixed into 400 μg beta-glucan (~200,000 Daltons) in 100 μl saline by oral gavage while control mice were given plasmid alone. Oral feeding was

done for 3 consecutive days (days 1, 2 and 3). 50 μl blood taken from tail vein were analysed by FCAS analysis after lysis of RBC and the % of GFP-expressing cells in the monocyte population were recorded. The mean ratio of % green cells in glucan verus no glucan groups (n = 4-9 mice per group) is presented in figure 9. Thoughout the 14 days of the experiment, % green monocytes in the no-glucan group remained stable at background levels. On the other hand, after day 1 of oral gavage, there was a consistent higher % of circulating green monocytes, which peaked around day 8. Since the GFP is not normally found in mouse monocytes, the presence of green cells is consistent with GFP protein expression following entry of the plasmid into the monocytes which circulate in the blood.

The experiment was repeated using barley β-glucan of higher molecular weight (~350,000 Daltons) with better gelling properties. In figure 10, similar kinetics was seen, with a higher percent of green cells that persisted from day 8 through day 11 (n=4 mice per group).

Presence of GFP mRNA was tested using quantitative reverse-transcription PCR analysis. Mice were fed with 50 μg pEGFP-c1 plasmid mixed into 400 μg high molecular weight (~350,000 Daltons) beta-glucan in 100 μl saline by oral gavage while control mice were given plasmid alone. 50 μl peripheral blood was used to extract total RNA, reverse transcribed and quantitative real-time PCR was performed using a modification of the method previously described.[4] The house keeping gene mouse GAPDH is used as internal control. Transcript level is calculated using a known GFP and GAPDH standard. Transcript units are calculated separately for GFP and GAPDH and results as a ratio of GFP over GAPDH. In figure 11, the mean RNA level (GFP/GAPDH) is expressed as a ratio of glucan versus no glucan groups (n=4 mice per group). GFP mRNA was detected up to day 10.

References for Example VI

1. Damge C, Aprahamian M, Marchais H, et al: Intestinal absorption of PLAGA microspheres in the rat. J Anat 189 (Pt 3):491-501, 1996

2. Jani P, Halbert GW, Langridge J, et al: Nanoparticle uptake by the rat gastrointestinal mucosa: quantitation and particle size dependency. J Pharm Pharmacol 42:821-6, 1990

3. Florence AT: The oral absorption of micro- and nanoparticulates: neither exceptional nor unusual. Pharm Res 14:259-66, 1997

4. Cheung IY, Lo Piccolo MS, Collins N, et al: Quantitation of GD2 synthase mRNA by real-time reverse transcription-polymerase chain reaction: utility in bone marrow purging of neuroblastoma by anti-GD2 antibody 3F8. Cancer 94:3042-8, 2002